Expansion of human autologous cytotoxic T lymphocytes on fixed target tumor cells

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Abstract

Human tumor specific cytotoxic T lymphocytes (CTL) were expanded on formalin-fixed autologous target tumor cells derived from glioblastoma multiforme. Growth response of the CTL restimulated with the fixed target cells was larger than those with live target cells. The results suggest that formalin-fixed tumor cells will be stable sources of tumor antigen for efficient autologous CTL expansion and be useful for adoptive immunotherapy of tumors.

Abbreviations: CTL – cytotoxic T lymphocytes; E/T ratio – effector/target ratio; LAK cells – lymphokine-activated killer cells; TIL – tumor-infiltrating lymphocytes

Introduction

Cytotoxic T Lymphocytes (CTL) are known to have higher specificity and killing activity against tumor cells than lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes (TIL) (Liu et al., 1995). Results of previous studies indicated that when autologous CTL could be induced, these would be more suitable than LAK or TIL for adoptive immunotherapy of tumors (Crowley et al., 1991; Wolfel et al., 1993; Tjoa and Kranz, 1994; Liu et al., 1996). However, considering that the minimum tumor size found in patients is practically 1-cm in diameter which is assumed to contain more than 10^9 tumor cells, CTL must be expanded to a considerably large number in order to be effective in tumor treatment. For repeated stimulation of CTL growth in the long-term expansion culture, sufficient number of target tumor cells should also be readily available (Crowley et al., 1991; Wolfel et al., 1993). However, primary culture of human tumor cells and establishment of the tumor cell lines are time-consuming and tedious processes, and not necessarily successful for every patient (Hay et al., 1994).

We have recently reported that, on formalin-fixed paraffin-embedded tumor sections which were a substitute for live tumor cells, human autologous CTL with high specificity and strong killing activity against the target tumor cells were inducible (Liu et al., 1996), since formalin fixation preserved the specific antigenicity of the target (Holden et al., 1986; Pollard and Holgate, 1987). To our regret, supply of tumor sections was also insufficient when the CTL grew to a considerable number, and therefore, we needed to restimulate CTL with anti-CD3 monoclonal antibody to maintain the culture (Liu et al., 1995). However, repeated stimulation with this antibody sometimes results in loss of cytotoxic activity of the CTL population (Muul et al., 1987). We observed this phenomenon in several maintenance cultures of human autologous CTL (Saijo, K., unpublished).

In clinical practice, on-time CTL administration in scheduled therapeutic courses is not necessarily kept because of unmatched timing of the target cell preparation, the growth of effector cells, and condition of the patient and the hospital. Rescheduling of the administration of CTL happens very frequently. Therefore,

conditioning of CTL with timely restimulation in a prolonged culture is essential for the repeated administration to a patient.

This timing adjustment problem could be solved if target tumor cells that have been previously fixed and supplied properly could also serve as useful source of tumor antigen available any time for further CTL stimulation. In the present study, we compared the stimulatory effect of live target tumor cells and fixed target tumor cells on the maintenance culture of human autologous CTL.

Materials and methods

Human glioblastoma cell line, TKB-1p, was provided from RIKEN Cell Bank. The cells were maintained in RHAM α medium (Kawai et al., 1992) containing 10% fetal bovine serum (FBS). Live target tumor cells were seeded into 6 well plates with 2 or 3 ml of the medium per well at a density of 2.5×10^4 cells/ml or 2.5×10^5 cells/ml, respectively, and were cultured until complete adherence of target cells. Immediately before use as live target cells for restimulation of the CTL, the tumor cells were subjected to X-ray irradiation at 50 Gy, and then the medium was replaced with RHAM α containing 1% human serum albumin (HSA). A part of the cultured live target tumor cells was fixed with 10% (v/v) formalin in Dulbecco's phosphate buffered saline (PBS) for 1hr at room temperature, washed once with 70% ethanol, three times with PBS, and twice with RHAM α , and then incubated overnight in 1% HSA-RHAM α . After replacing with fresh medium, the fixed tumor cells were supplied for restimulation of the CTL.

Human CTL (TKB-1p-CTL) specific to the autologous target TKB-1p were also provided from RIKEN Cell Bank. These CTL were initially generated on fixed TKB-1p cells (Tsurushima et al., submitted for publication) and maintained in the RHAM α medium containing 5% autologous plasma, recombinant human interleukin(IL)-1 β (167 U/ml), IL-2 (67 U/ml), IL-4 (67 U/ml), and IL-6 (134 U/ml) in 6-well plates of which wells were pre-coated with mouse anti-human CD3 monoclonal antibody (OKT-3, Nichirei Co., Tokyo) at a concentration of 0.05 μ g/ml and 0.2 ml/cm². The antibody stimulation was done bi-weekly by transferring the CTL to the plates freshly precoated with the anti-CD3 antibody. These IL-1 β , IL-2, IL-4, and IL-6 were kindly provided by Ohtsuka Pharmaceuticals, Co. (Tokushima, Japan), Take-

da Chemical Industries, Co. Ltd. (Tokyo), Ono Pharmaceutical Co. (Osaka), and Ajinomoto Co. (Tokyo), respectively.

A gastric cell line GT3TKB was submitted as an allogeneic control when growth response of the CTL was confirmed against autologous live target TKB-1p cells. GT3TKB was obtained from routine cultures in the RIKEN Cell Bank. Serological typing of major-histocompatibility complex of GT3TKB showed to be HLA-A24 while the autologous TKB-1p was HLA-A11,26 (data not shown). For observation of the growth response of the CTL, the target tumor cells were seeded in a 24-well culture plate and cultured until the cells reached to a confluency. To the culture, after the X-ray irradiation described as above and removal of the tumor-cell culture medium, 6.8×10^5 of the CTL suspended in one ml of RHAM α medium containing 5% autologous plasma and the cytokines were added. The medium was replaced every 3 days. After the co-culture, the CTL were suspended by gentle pipetting and counted in a hemocytometer. Since the CTL killed all of TKB-1p cells within a day of the co-culture, approximately the same number of TKB-1p cells per well were supplied again at 7th day in the co-culture. GT3TKB cells were unaffected during the co-culture. Observations were made tetraplicated for a point.

After we have received *again* the CTL for further experiments, the 5% autologous plasma in the culture medium was replaced with 1% HSA; IL-4 and IL-6 were excluded; the CTL were restimulated periodically (7–10 day intervals) with X-ray irradiated live target TKB-1p cells at an effector/target (E/T) ratio of 10. However, in the experiments (Figs. 2 and 3), we used the 'starving' CTL which had not been restimulated with the live target tumor cells for at least 7 days.

Results and discussion

The TKB-1p-CTL have been known to kill the autologous target TKB-1p cells, but not allogeneic renal carcinoma cells nor gastric carcinoma cells (Tsurushima et al., in submission). Specific growth response of the CTL was confirmed against TKB-1p cells but not against allogeneic gastric tumor GT3TKB cells (Fig. 1). In this particular experiment, the control CTL grew slightly for 6 days with no target cells, then the number of CTL decreased gradually.

As shown in Fig. 2, we have stimulated growth of the CTL with both live target TKB-1p cells previously irradiated with X-ray and fixed target TKB-1p cells at

Figure 1. Specific growth response of TKB-1p-CTL to the autologous target tumor cells, TKB-1p. TKB-1p-CTL were added at the day 0 to the live target tumor cells (autologous TKB-1p or allogeneic GT3TKB) previously irradiated with X-ray or without target cells (Control, no target cells). Vertical bars at the 3rd day represent standard deviation of the mean value of triplicate observations for a point. In all the other observed points, the deviation was so small that they were within the size of open marks.

an E/T ratios of 1 and 10. The number of control CTL gradually decreased after culturing for 2 days. This control culture consisted of all the components except the target cells. With the fixed target TKB-1p cells, the CTL grew continuously at E/T ratios of 1 and 10 with the mean doubling times of 95 and 119 h, respectively. With the X-ray irradiated live target TKB-1p cells, however, growth rates of the CTL were substantially the same at both E/T ratios of 1 and 10. The mean doubling times were 138 and 106 hr, respectively. These values were similar to that of the CTL stimulated with the fixed target tumor cells at the E/T ratio of 10 and larger than that of the CTL stimulated with the fixed target cells at the E/T ratio of 1.

Since all the live target cells disappeared within 4 days of culture of the CTL at both E/T ratios of 1 and 10, it was possible that the stimulatory signal may have been insufficient to last for a long time period. On the other hand, the CTL cultured on the fixed target cells may have received continuous stimulation from the target cells. We have tried to stimulate the CTL with

the fixed target cells for a term which was adjusted to the remaining term of the live target cells. As shown in Fig. 3a, the CTL were cultured on the fixed target cells for only 4 days at the first stimulation (indicated with the closed under-bars in Fig. 3a), then continued to culture without the fixed target cells. At the E/T ratio of 1, the CTL stimulated with live target cells continued to grow for 6 days, while the CTL stimulated with fixed target cells grew only for 4 days, then began dying (Fig. 3a). At the second stimulation, growth of the CTL stimulated with fixed target TKB-1p cells at the E/T ratio of 1 lasted for 10 days. The growth rate was close to that of the CTL stimulated with live target cells. After the third stimulation in this particular experiment, the CTL stimulated with fixed target cells grew faster than the CTL stimulated with live target cells for the first 3 days, and then grew at the same rate. The cumulative growth curves of CTL (Fig. 3b) revealed that, under the condition in which the same term of stimulation was applied with both live and

Figure 2. Continuous stimulation of CTL growth with fixed tumor cells. Arrows indicate the restimulation with X-ray irradiated live or formalin-fixed TKB-1p cells at the E/T ratio indicated. A point indicates mean of duplicated observations. Control culture consisted of all the components except target cells.

fixed target cells, live target cells were apparently more efficient than the fixed target cells.

We assumed that efficient presentation of surface antigenic molecules and costimulatory molecules to the CTL (Schwartz, 1992; David et al., 1996) may have occurred in the live target cells because of their membrane fluidity. Such fluidity was absent in the fixed target cells, therefore, resulting in less short-term efficiency in CTL growth stimulation compared to live target cells (Fig. 3). However, continuous long-lasting contact of the fixed target cells to the CTL contributed the larger growth rate of the CTL as shown in Fig. 2.

The present study suggests that CTL are continuously expandable on formalin-fixed autologous tumor cells as well as on live autologous established tumor cell line. Even though we have failed to establish the autologous tumor cell line, many investigators may have found target tumor cells available in small pieces of the dissected tumor tissue, in the fluid of thoracic cavity and ascites, or in quiescent primary cultures. If

they are fixed and supplied properly, these cells could also serve as source of tumor antigen available any time for further CTL stimulation. These fixed antigenic resources will provide for an efficient strategy for adoptive immunotherapy of tumors.

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Figure 3. Discontinuous stimulation of CTL growth with target tumor cells. a) CTL were co-cultured with fixed tumor cells in the term shown with black under-bars or without fixed tumor cells in the term shown with dotted under-bars. Arrows indicate the restimulation with X-ray irradiated live or formalin-fixed TKB-1p cells at the E/T ratio indicated. A point indicates mean of duplicated observations. b) Cumulative cell number of Fig. 3a.

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